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# Fluorescent proteins and chromoproteins in phylum: Cnidaria

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## Abstract

Green fluorescent protein was first discovered in *Aequorea victoria.* Its significance in a continually expanding range of scientific applications led to the discovery of an abundance of homologous fluorescent proteins and non-fluorescent chromoproteins in a variety of species in the phylum Cnidaria. The document reviews the various proposed hypotheses on the biological functions and biochemistry of fluorescent proteins and chromoproteins, wherein a full resolution remains elusive and is the subject of on-going debate. Mutagenesis has provided novel variants and insights into the relationship between the spectral characteristics and chromophore structures of fluorescent proteins that encompass the visible spectrum. Fluorescent proteins as genetically encoded reporters have revealed important aspects of cellular biology and physiology that would have been unobtainable using traditional *in vitro* methods. The development of fluorescent proteins has opened up numerous possibilities for novel imaging techniques using living cells.

Keywords: Green fluorescent proteins, fluorescent proteins, chromoproteins, Cnidaria, Anthozoa, Hydrozoa

## Introduction

Green fluorescent protein (GFP) was initially discovered as a component of the bioluminescent system in *Aequorea victoria* (Shimomura 2005). Blue light from the calcium binding protein aequorin is absorbed and converted into green fluorescence by the GFP chromophore via bioluminescent resonance energy transfer (Reid & Flynn 1997). Cloning in *E. coli* confirmed the GFP gene contains all the necessary information for post-translational synthesis of the chromophore without accessory co-factors, external enzymatic catalysis or substrates other than molecular oxygen (Prasher *et al* 1992).

The significance of GFPs in fluorescent bio-imaging led to the discovery of an abundance of fluorescent proteins (FPs), chromoproteins (CPs) and GFP-like domains (G2FP) that form a large family of homologues in Cnidaria and Bilateria species (Miyawaki 2002). Phylogenetic analysis of Cnidaria FPs and G2FP extracellular matrix proteins identified in the phylum Nematoda and Chordata, indicate lineage divergence from a common ancestor. Copepoda FPs are similar to Cnidaria FPs which suggests a gene duplication event preceding the separation of Bilateria and Cnidaria. This implies G2FP and FP genes were present in the common ancestor (Shagin *et al* 2004).

Analysis of Cnidaria genome sequences reveal a ubiquitous protein family from several Hydrozoa and over a hundred Anthozoa species, comprised of around 220-240 amino acid residues approximately 25-30 kDa in size (Alieva *et al* 2008). Mutagenesis has created novel spectral variants across the entire visible spectrum and provided insights into the relationship between FP structure and excitation and emission characteristics induced by amino acid residues within or in close proximity to the different chromophore structures that have been identified (Zimmer 2002).

## Biological functions

The biological function of FPs in Cnidaria is still the subject of much debate. Bioluminescence via GFP provides a more visible signal than blue light from aequorin or luciferase against a blue aqueous environment. Bioluminescence may therefore impart protection from predation, function to lure prey, attract symbionts or potential mates (Chudakov *et al* 2010). Bioluminescence has also been speculated to be an evolutionary relic of a primitive proton pump involved in generating energy or in regenerating luciferin and coelenterazine (Shinobu *et al* 2010).

FPs and CPs are suggested to provide photoprotection in host tissues and symbionts, thereby limiting damage from excessive light exposure; a hypothesis supported by low densities of FPs in bleaching susceptible taxa and up regulation of FP expression in high light conditions (Baird *et al* 2008). In some species FPs may serve as conduits which direct light to zooxanthellae to enhance light availability (Verkhusha & Lukyanov 2004). In addition, *in vitro* experiments reveal immunological roles in compromised tissues and significant reactive oxygen species scavenging activity (Palmer *et al* 2009). Based on the external proton collecting antennae and internal proton wire identified by Shinobu *et al* (2010), it is possible that proton transport is involved in mediating pH dependent processes, such as calcium carbonate deposition in Scleractinia. FPs may also function as light driven electron donors, although extensive experimentation is necessary to elucidate their biological roles.

## Molecular properties of fluorescent proteins and chromoproteins

FPs and CPs have a β-barrel structure made up of 11 β-strands forming a cylinder with an α-helix running through the central axis, stabilised by multiple non-covalent interactions between the amino acid residues (Yang *et al* 1996). The chromophore is situated in the middle of the α-helix and is enclosed by the β-barrel, thus protecting the chromophore from the external environment (Phillips Jr. 1997). The spectral diversity of FPs arise from interactions between the chromophore, the amino acids within the microenvironment and from the different chromophore conformations formed via alternative pathways (Labas *et al* 2002). Large spectral shifts are attributed to differences in covalent structures and the extent of the π-orbital conjugation of the chromophore (Shaner *et al* 2007).

### Green Fluorescent Proteins

Wild-type (wt)GFP from *A. victoria* has been studied and developed extensively, revealing the induction of fluorescence through auto-catalytic modifications of the tripeptide that make up the 4-(p-hydroxybenzylidene) imidazolidin-5-one chromophore (Ormö *et al* 1996). The conventional chromophore maturation model proposed by Tsien (1998) begins with imidazolinone ring formation from the nucleophilic attack of the amide nitrogen of Gly67 on the carbonyl carbon of Ser65 followed by dehydration. Dehydrogenation of the α-β carbon bond of Tyr66 by oxygen results in a conjugated π-system with a planar-cis two ring conformation composed of the tyrosine phenol and imidazolinone heterocycle (Brejc *et al* 1997).

The cyclization of the chromophore is mediated by the minimal steric hindrance of glycine which is highly conserved as the third amino acid in the chromophore sequence in all natural and engineered FPs that retain fluorescence (Stepanenko *et al* 2008). Tyrosine is also conserved as the central amino acid among all natural FPs while the preceding amino acid can vary (Chudakov *et al* 2010). An alternative chromophore formation mechanism based on density functional calculations suggests dehydration of Tyr66 prior to cyclization is more energetically favourable, with reaction energies close to thermo-neutral while forming a more stable intermediate. Prior dehydration is also structurally advantageous as it provides a shorter distance for cyclization between the carbonyl carbon of Ser65 and the amide nitrogen of Gly67 (Siegbahn *et al* 2001).

wtGFP has a major absorption peak at 395 nm and a minor peak at 475 nm, with emission peaks of 508 nm and 503 nm respectively (Tsien 1998). The different absorption peaks are induced by an excited state proton transfer upon irradiation. The 395 nm absorption is attributed to the neutral form while absorption at 475 nm to an anionic form following the transfer of a phenolic proton from Tyr66 to the carboxyl oxygen of Glu222 (Shinobu *et al* 2010). The peak at 475 nm has a small shoulder on the edge of the red spectrum and is suggested to be caused by a zwitterionic chromophore, although a Raman spectroscopy study and lack of experimental evidence challenges the validity of the theory. Various photoisomerization models have been proposed and partially validated but the exact mechanism remains to be fully resolved (Zimmer 2002). Commonly used GFP variants with a Ser65 to Thr65 substitution undergo deprotonation on the phenolic side chain to yield an anionic green chromophore with a single absorbance and emission maximum of ~480 and ~510 nm respectively (Reid & Flynn 1997).

A hydrogen bond network is formed by several key amino acids located close to the chromophore, including Arg96 which promotes protein backbone cyclization and Glu222 which is associated with catalytic activity. Both amino acids are highly conserved in all natural FPs, however site directed mutagenesis have shown that proper protein folding and fluorescence can still be achieved despite substitutions. Side-chains of residues 148, 165, 167 and 203 are in contact with Tyr66 and determine spectral properties, protonation state, spatial conformation and rotational freedom of the chromophore (Stepanenko *et al* 2008).

GFP modifications have produced variants emitting in the blue (BFP), cyan (CFP) and yellow (YFP) spectrum. Useful GFP derivatives in imaging applications are mEGFP, mEmerald and sfGFP (Table 1) (Stepanenko *et al* 2004; Shaner *et al* 2007). Other GFPs have also been developed from various species, such as the CP from *Aequorea coerulescens* (Gurskaya *et al* 2003) and GFP from *Galaxeidae* sp. (Karasawa *et al* 2003), with many more to be discovered that will extend the existing FP palette (Millwood *et al* 2008; Day & Davidson 2009).

**Cyan Fluorescent Proteins**

ECFP and mCerulean are derived from *A. Victoria* GFP and have a Trp66 centred chromophore (Table 1) (Shaner *et al* 2005). The naturally occurring teal-coloured FP from soft coral *Clavularia* sp. has a Ser66-Tyr67-Gly68 chromophore, developed into a monomer labelled mTFP1 (Table 1) (Topol *et al* 2010). Other commercially available CFPs include AmCyan1 from *Anemonia majano* and Midori-ishi Cyan (MiCy) from stony coral *Acropora* sp. (Karasawa *et al* 2004).

**Blue Fluorescent Proteins**

BFP with a His66 substitution was one of the first *A. victoria* GFP-derived spectral variants (Heim *et al* 1994). Improvements have been attained through mutagenesis yielding enhanced versions such as EBFP2 (Table 1) (Shaner *et al* 2007). A Phe66 substitution produced a violet coloured variant called Sirius, with excitation and emission peaks of 355 nm and 424 nm respectively (Chudakov *et al* 2010). Red FPs (RFPs) have a transient blue intermediate. Introducing site specific mutations that prevent the maturation of the chromophore has created several BFP variants, with the most promising being mTagBFP2 based on superior brightness, faster maturation and higher pH stability compared to Hydrozoa derived BFPs (Subach *et al* 2011).

**Yellow Fluorescent Proteins**

Probes often used in the yellow spectral class are engineered from *A. victoria* GFP, such as EYFP and YPet, mCitrine and mVenus (Table 1) (Shaner *et al* 2005). Few natural YFPs have been identified, and phiYFP with excitation and emission peaks of 525 nm and 537 nm respectively, is to date the only natural Hydrozoa YFP discovered in *Phialidium* sp. A Leu64 substitution to enhance folding and a Tyr203 substitution to produce yellow fluorescence in *A. victoria* GFP is also observed in phiYFP (Day & Davidson 2009). Yellow fluorescence arises from the ππ-stacking of Tyr66 and Tyr203.

ZsYellow was discovered in Anthozoa button polyp *Zoanthus* sp., with excitation and emission peaks of 528 and 538 nm, respectively (Verkhusha & Lukyanov 2004). The Lys66-Tyr67-Gly68 chromophore is a DsRed-type derivative (Alieva et al 2008) and constitutes a three ring moiety. A tetrahydropyridine ring is formed from the peptide

**Table 1:** Physical properties of useful fluorescent proteins (Adapted from Shaner *et al* 2004 and 2007).

| Protein | Spectral Class | Excitation Peak (nm) | Emission Peak (nm) | Association State | Brightness* | Chromophore amino acids |
|---|---|---|---|---|---|---|
| EBFP2 | Blue | 383 | 448 | Weak dimer | 18 | Ser-His-Gly |
| ECFP | Cyan | 433/445 | 475/503 | Weak dimer | 13 | Thr-Trp-Gly |
| mCerulean | Cyan | 433/445 | 475/503 | Monomer | 27/24 | Thr-Trp-Gly |
| mTFP1 | Cyan-green | 462 | 492 | Monomer | 54 | Ala-Tyr-Gly |
| mEGFP | Green | 488 | 507 | Monomer | 34 | Thr-Tyr-Gly |
| mEmerald | Green | 487 | 509 | Monomer | 39 | Thr-Tyr-Gly |
| sfGFP | Green | 485 | 510 | Weak dimer | 54 | Thr-Tyr-Gly |
| EYFP | Yellow | 514 | 527 | Weak dimer | 51 | Gly-Tyr-Gly |
| mVenus | Yellow | 515 | 528 | Monomer | 53 | Gly-Tyr-Gly |
| mCitrine | Yellow | 516 | 529 | Monomer | 59 | Gly-Tyr-Gly |
| YPet | Yellow | 517 | 530 | Weak dimer | 80 | Gly-Tyr-Gly |
| mOrange | Orange | 548 | 562 | Monomer | 49 | Thr-Tyr-Gly |
| mKO | Orange | 548 | 559 | Monomer | 31 | Cys-Tyr-Gly |
| tdTomato | Orange | 554 | 581 | Tandem dimer | 95 | Met-Tyr-Gly |
| TagRFP | Orange | 555 | 584 | Monomer | 48 | Met-Tyr-Gly |
| mRFP1 | Red | 584 | 607 | Monomer | 12.5 | Gln-Tyr-Gly |
| mCherry | Red | 587 | 610 | Monomer | 17 | Met-Tyr-Gly |
| mKate | Far-red | 588 | 635 | Monomer | 15 | Met-Tyr-Gly |
| mPlum | Far-red | 590 | 649 | Monomer | 3.2 | Met-Tyr-Gly |

*Brightness is the product of the molar extinction coefficient and quantum yield $(mM \times cm)^{-1}$

backbone cleavage and cyclization of Lys66 with its own α carbon (Day & Davidson 2009).

**Orange Fluorescent Proteins**

Few FPs emit in the orange spectrum and one of the first natural orange FPs to be identified was a tetramer isolated from mushroom coral *Fungia concinna,* commercially available as Kusabira Orange (KO). The monomer mKO was developed by substituting twenty amino acids and adding seven amino acids to the N-terminus (Table 1) (Karasawa *et al* 2004). The Cys66-Tyr67-Gly68 chromophore of mKO matures similarly to DsRed, although Cys66 undergoes a further cyclization with its carbonyl carbon to produce a three ring chromophore featuring a 2-hydroxy-3-thiazoline motif. DsRed derived mOrange with a Thr66-Tyr67-Gly68 chromophore, follows the same maturation as mKO but yields an oxazole heterocycle from the cyclization of Thr66 (Wiedenmann *et al* 2009).

**Red Fluorescent Proteins**

The impetus for the development of RFPs is to reduce cellular autofluorescence, therefore permitting deeper probe detection and longer periods of imaging from decreased cellular phototoxicity when using longer wavelengths (Day & Davidson 2009). Chromophores which fluoresce in the red spectrum have a green intermediate and can be classified as DsRed-type or Kaede-type (Fields & Matz 2010). Kaede-type FPs are associated with a few species from Anthozoa Class: Alcyonaria and Zoantharia. The majority of Anthozoa RFPs possess the DsRed-like chromophore (Alieva *et al* 2008).

Kaede is from *Trachyphyllia geoffroyi* and has the chromophore His62-Tyr63-Gly64; typical of photoconvertible FPs which include EosFP from *Lobophyllia hemprichii* and dendGFP from *Dendronephthya* sp. (Gurskaya *et al* 2006). Fluorescence can be converted irreversibly from green to red by ultraviolet irradiation, which induces the cleavage of the protein backbone between the amide nitrogen and α carbon of His62 followed by double bond formation between the α and β carbon (Verkhusha & Lukyanov 2004).

The DsRed tetramer is the first extensively characterized RFP from the soft coral *Discosoma* sp. (Shaner *et al* 2004), with a Gln66-Tyr67-Gly68 chromophore in a planar-cis conformation common to most RFPs (Yarbrough *et al* 2001). Other Anthozoa RFPs can contain methionine, glutamine, threonine, cysteine or glutamate in the 66$^{th}$ position (Verkhusha & Lukyanov 2004). The conventional model for the maturation of the DsRed chromophore begins with heterocycle formation similar to GFP (Gross *et al* 2000). The amide nitrogen of Gly68 forms a heterocycle with the carbonyl carbon of Gln66, and deprotonation of the tyrosine phenol results in the green anionic intermediate with excitation and emission peaks of 480 nm and 500 nm respectively (Stepanenko *et al* 2008). The subsequent oxidation of the α carbon and nitrogen bond of Gln66 accounts for the red fluorescence of the mature protein, with excitation and emission peaks of 558nm and 583nm, respectively (Verkhusha *et al* 2004).

The heterocycle of the DsRed chromophore, with p-hydroxybenzylidene and acylimine substituents (Shaner *et al* 2004), is postulated to be an extension of the GFP-like core based on initial green fluorescence in immature wild-type and mutant proteins. Green fluorescence gradually decreases relative to an increase in red

fluorescence (Zimmer 2002). However, more recent studies provide alternative pathways because it was identified that a portion of chromophores retain green fluorescence, which is progressively suppressed by intratetramer fluorescence resonance energy transfer (FRET) from the slower forming red chromophore (Strack *et al* 2010).

Verkhusha *et al* (2004) suggested oxidation of the α-β carbon bond of a protonated Tyr67 creates a neutral chromophore which accounts for the observed transient blue intermediate. Maturation can then proceed via two exclusive pathways resulting in either green or red fluorescence. Deprotonation of the Tyr67 phenol forms the irreversible green anionic chromophore. Hydrogen bonding with Lys163 ensures the Tyr67 phenol oxygen remains charged thus accounting for its stability. The neutral chromophore can mature to the red chromophore because the α carbon of Gln66 carries a partial negative charge while its hydrogen carries a partial positive charge. This polarization facilitates the formation of an unstable carbanion intermediate where the α carbon becomes negatively charged from deprotonation, resulting in a double bond between the α carbon and nitrogen of Gln66.

Strack *et al* (2010) proposed a novel branched pathway for DsRed maturation as an alternative to the aforementioned irreversible anionic chromophore model. The pathway involves cyclization followed by oxidation, resulting in an equilibrium mixture of a hydroxylated cyclic imine and a cyclic imine which forms the branching point of the pathway. Dehydration of the hydroxylated cyclic imine and a subsequent deprotonation creates the anionic green chromophore. Oxidation of the cyclic imine yields the transient blue intermediate which is then hydroxylated and dehydrated to form a neutral chromophore, and finally deprotonated to the anionic red chromophore.

Unlike Hydrozoa FPs, Anthozoa FPs mature efficiently at 37 °C, possibly due to adaptations to shallow and warm tropical environments, which makes them ideal tags for *in vivo* imaging (Snap 2009). Mutagenesis has extended the emission range of Anthozoa FPs to 600-655 nm and eliminated the typical tetramerization tendencies which may have also arisen from adaptations to high light conditions (Piatkevich *et al* 2010). The directed evolution of DsRed derivative mRFP1 has produced a group of monomeric FPs and a tandem dimer, collectively called ‘mFruits’, which span the emission range of 540-610 nm (Table 1) (Shu *et al* 2006).

Uncommon chromophores in the planar-trans and nonplanar-trans conformations were initially identified in *Entacmaea quadricolor* (eqFP611) and *Montipora efflorescens* (Rtms5), respectively. The Met63-Tyr64-Gly65 chromophore of eqFP611 forms a 5-[(4-hydroxyphenyl) methylene]-imidazolinone motif with absorbance and emission peaks of 559 and 611 nm, respectively (Petersen *et al* 2003).

asCP from *Anemonia sulcata* received a lot of interest due to its ability to be reversibly switched on and off. Green light irradiation induces a small amount of fluorescence which can be quenched with blue light (Lukyanov *et al* 2000). Initial proposals to the asCP structure was a truncated version of the β-barrel motif (Wiedenmann *et al* 2000) or a fragmentation that splits the structure into 8 kDa and 20 kDa sections (Martynov *et al* 2001). Chudakov *et al* (2003) proposed a cis-tran isomerisation or chromophore turning mechanism. asCP crystal structure revealed

the Met63-Tyr64-Gly65 chromophore has a nonplanar-trans p-hydroxybenzylideneimidazolinone moiety, which is fragmented between the Cys62 carbon and Met63 nitrogen during maturation (Wilmann *et al* 2004). More importantly, the 'kindling' effect was transferred to other CPs with amino acids substitutions Ala148, Ser165 and His203 observed in wild-type asCP. Mutagenesis of asCP resulted in the brighter KFP1 with excitation and emission peaks of 580 nm and 600 nm respectively (Chudakov *et al* 2003). The most red-shifted FP was engineered from a CP identified in *Actinia equina* (AQ143), with excitation and emission peaks of 595 nm and 655 nm, respectively (Shkrob *et al* 2005).

## Molecular and Cell Biology Applications

Non-invasive FP probes have contributed greatly to an expanding range of scientific applications. The gene that encodes the FP is hybridized to the gene of interest, and excitation using a particular wavelength of light enables monitoring of expression, movement, localization, interaction and activity (Chudakov *et al* 2005). The myriad of FPs with different physical and spectral properties permit their simultaneous expression in cells, tissues and in a variety of organisms.

Overcoming oligomerization has reduced non-specific aggregation and improved FPs as fluorescent labels (Bulina *et al* 2003). Organelles and proteins can be tagged and observed in time-lapse, in three dimensions and with high resolution, to reveal temporal and spatial information on intracellular processes that govern the cell and its constituents. To measure gene expression, the FP gene is cloned under the control of a promoter and the activity of the promoter can be measured relative to the amount of fluorescence. In addition, FP timers which change colour over time provides a new approach for monitoring time related promoter/protein activity (Piatkevich *et al* 2010).

The development of FPs which can change photophysical properties make valuable tools as optical highlighters and opens up the possibilities for developing novel microscopy techniques. Optical highlighters allow the direct, controlled and rapid activation of a distinct population of FPs in a cell (Piatkevich & Verkhusha 2010). Photoactivatable FPs are capable of increasing fluorescence emission upon irradiation of a specific wavelength of light. Photoconvertible FPs are able to change fluorescence from one colour to another, while photoswitchable FPs can be switched from a non-fluorescent to a fluorescent state and *vice versa*.

FPs are useful in determining cellular architecture, cell lineage during development (Toshiki *et al* 2010) and elucidating infection mechanics of viruses and bacteria (Banfield *et al* 2003; Betzig *et al* 2006; Zamboni *et al* 2006). Fluorescent imaging in murine and zebra fish models help evaluate disease progression and inhibition by drugs, consequently presenting insights in disease development and therapy (Hoffman 2005; Lieschke & Currie 2007). FPs with high quantum yield and photostability have enhanced the sensitivity of kinetic microscopy techniques using live cells. FRET is the non-radiative transfer of energy from a donor to an acceptor FP. It is used to highlight interactions of various proteins, such as integrins fused with FPs of different colours, to determine dynamic modulation (Malkani & Schmid 2011; Carman 2012). An alternative method for monitoring protein interactions is fluorescence correlation spectroscopy (FCS). FCS detects temporal fluctuations of a fluorescence signal from one or more FPs with different spectral emissions within a defined focal volume (Ray *et al* 2010; Wu *et al* 2011). Quantitative information on

concentration and diffusion rates of molecules can therefore be obtained. For example, Dieteren *et al* (2011) demonstrated the concentration and decreased diffusion of monomeric FPs within the mitochondrial matrix using FCS.

Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are widely used techniques to study protein mobility. FRAP involves photobleaching a region of interest while observing the migration of unbleached proteins into the bleached area (Lippincott-Schwartz & Patterson 2003). Membrane continuity and mobility of FP labelled proteins within the endoplasmic reticulum, the nucleus and other organelles have been defined using FRAP (Reits & Neefjes 2001). FLIP is a complementary approach to FRAP and involves photobleaching a region of interest repeatedly while collecting images of the entire cell. Fluorescence loss over time provides information on structural continuity and permeability (Davies *et al* 2010).

## Conclusion

The biological function of FPs is not fully understood and probably varies between species. Understanding the biological purpose of FPs in their natural hosts may be complicated but will provide important insights to their evolution and the ecology of marine organisms. This unknown function necessitates extensive investigation because it may have an effect on the physiology of recombinant expression systems and might limit the use of FPs as a tool kit for imaging.

The different physical and chemical properties of FPs must be considered prior to its use because none of the available FPs is equally well suited for all imaging purposes. Factors and mechanisms that facilitate chromophore maturation remain unclear despite extensive mutagenesis experiments. Further investigations combined with high resolution structure mapping, spectroscopy and computer modelling have expounded on existing theories and will eventually elucidate the molecular processes that underlie the photophysical behaviour of FPs. The knowledge obtained will be essential in developing ideal fluorescent labels with improved brightness, photostability, pH tolerance, maturation rate and photoswitching.

Using multi-coloured FPs have revealed important aspects of cellular biology that would have been unobtainable using traditional *in vitro* methods. Significant progress has been attained in expanding the FP palette and emerging high resolution microscopy techniques will complement future developments in experimental investigations.

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